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Molecular Determination of Point Mutation Haplotypes in the Dihydrofolate Reductase and Dihydropteroate Synthase of *Plasmodium falciparum* in Three Districts of Northern Tanzania

Richard J. Pearce,[1]* Chris Drakeley,[1,2] Daniel Chandramohan,[1] Frank Mosha,[2,3] and Cally Roper[1]

Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, WC1E 7HT, United Kingdom,[1] and Kilimanjaro Christian Medical Centre[3] and The Joint Malaria Programme (JMP)[2] Moshi, Tanzania

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The antimalarial combination of sulfadoxine and pyrimethamine (SP) was introduced as first-line treatment for uncomplicated malaria in Tanzania during 2001 following 18 years of second-line use. The genetic determinants of in vitro resistance to the two drugs individually are shown to be point mutations at seven sites in the dihydrofolate reductase gene (*dhfr*) conferring resistance to pyrimethamine and five sites in the dihydropteroate synthase (*dhps*) gene conferring resistance to sulfadoxine. Different combinations of mutations within each gene confer differing degrees of insensitivity, but information about the frequency with which allelic haplotypes occur has been lacking because of the complicating effects of multiple infection. Here we used a novel high-throughput sequence-specific oligonucleotide probe-based approach to examine the present resistance status of three *Plasmodium falciparum* populations in northern Tanzania. By using surveys of asymptomatic infections and screening for the presence of all known point mutations in *dhfr* and *dhps* genes, we showed that just five *dhfr* and three *dhps* allelic haplotypes are present. High frequencies of both triple-mutant *dhfr* and double-mutant *dhps* mutant alleles were found in addition to significant interregional heterogeneity in allele frequency. In vivo studies have shown that the cooccurrence of three *dhfr* mutations and two *dhps* mutations in an infection prior to treatment is statistically predictive of treatment failure. We have combined data for both loci to determine the frequency of two-locus genotypes. The triple-*dhfr*/double-*dhps* genotype is present in all three regions with frequencies ranging between 30 and 63%, indicating that treatment failure rates are likely to be high.

Sulfadoxine-pyrimethamine (SP) has now replaced chloroquine as the first-line curative antimalarial in much of East Africa. The earliest reports of emerging SP resistance in Africa were from Muheza district in Tanzania during 1994 and 1995 (32, 37). SP remains in use throughout Tanzania and was recently officially adopted as the national first-line treatment for nonsevere *Plasmodium falciparum* malaria. It is now a priority to learn how widespread genetic determinants of SP resistance currently are in the larger regions of northern Tanzania.

To investigate this issue, we have carried out a population-based genetic analysis of *P. falciparum* in the North and South Pare Mountains and Hai district, which are distinct geographical areas with their own microclimates and tribes. There had been no malaria research in these districts since 1965 until recent work showed relatively low levels of transmission, with an estimated entomological inoculation rate of 24 infective bites/person/year in Hai district (C. Drakeley and D. Chandramohan, unpublished data) compared with an entomological inoculation rate in the range of 34 to 405 infective bites/person/year in Muheza district (14).

It is widely understood that people self-treat with antimalarial drugs, which can be freely purchased (24); as a consequence, a proportion of people attending health facilities with signs and symptoms of malaria may have had recent prior exposure to drugs. In this study we have analyzed material from community surveys of asymptomatic infections. These data, we believe, are less subject to bias due to prior drug selection and are therefore representative of the parasite population at large.

Resistance to SP is associated in vitro with a series of substitutions within the active site of target enzymes of the folate biosynthesis pathway, dihydropteroate synthase (DHPS) (4, 38) and dihydrofolate reductase (DHFR) (9, 29, 36), and this has been demonstrated through laboratory-based in vitro sensitivity tests and transfection experiments with DHFR with respect to pyrimethamine (43, 44) and DHPS with respect to sulfadoxine (39, 40, 42). The sequence changes coding for substitutions that are naturally occurring worldwide are summarized in Table 1. A Ser-to-Asn substitution at codon 108 of DHFR decreases sensitivity to pyrimethamine 100-fold (43) Additional substitutions at codons 51 (N51I), 59 (C59R), and 164 (I164L) progressively increase levels of resistance to pyrimethamine (15, 43). Isolates containing all four substitutions have been found in South America and southeast Asia but have yet to be reported in Africa (21, 25, 41). A total of 14 DHPS substitutions at five sites have been characterized worldwide, of which 6 have been recorded in Africa, with A437G and

* Corresponding author. Mailing address: Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel St., London WC1E 7HT, United Kingdom. Phone: (44) 207 927 2416. Fax: (44) 207 636 8739. E-mail: Richard.Pearce@lshtm.ac.uk.

TABLE 1. Summary of each of the sites at which an SNP occurs which is known to be associated with SP resistance and the oligonucleotide probe designed to detect it

Probe	Amino acid	Probe sequence[a]
DHFR		
Codon 16		
A*[b]	Ala	CC ATA TGT **GCA** TG T TGT A
S	Ser	CC ATA TGT **TCA** TG T TGT A
V	Val	CC ATA TGT **GTA** TG T TGT A
Codons 50 and 51		
CN*	Cys Asn	TGG AAA **TGT AAT** TCC CTA
CN2*	Cys Asn	TGG AAA **TGT AAC** TCC CTA
RN	Arg Asn	TGG AAA **CGT AAT** TCC CTA
RN2	Arg Asn	TGG AAA **CGT AAC** TCC CTA
RI	Arg Ile	TGG AAA **CGT ATT** TCC CTA
CI	Cys Ile	TGG AAA **TGT ATT** TCC CTA
Codon 59		
C*	Cys	AA TAT TTT **TGT** GCA GTT A
R	Arg	AA TAT TTT **CGT** GCA GTT A
Codon 108		
N	Asn	A AGA ACA **AAC** TGG GAA AG
S*	Ser	A AGA ACA **AGC** TGG GAA AG
T	Thr	A AGA ACA **ACC** TGG GAA AG
Codon 140		
V*	Val	AT GAA GAT **GTT** TAT ATC A
L	Leu	AT GAA GAT **CTT** TAT ATC A
Codon 164		
I*	Ile	GT TTT ATT **ATA** GGA GGT T
L	Leu	GT TTT ATT **TTA** GGA GGT T
DHPS		
Codons 436 and 437		
SA*	Ser Ala	GAA TCC **TCT GCT** CCT TTT
SG	Ser Gly	GAA TCC **TCT GGT** CCT TTT
FA	Phe Ala	GAA TCC **TTT GCT** CCT TTT
FG	Phe Gly	GAA TCC **TTT GGT** CCT TTT
AA	Ala Ala	GAA TCC **GCT GCT** CCT TTT
AG	Ala Gly	GAA TCC **GCT GGT** CCT TTT
CA	Cys Ala	GAA TCC **TGT GCT** CCT TTT
Codon 540		
K*	Lys	ACA ATG GAT **AAA** CTA ACA
E	Glu	ACA ATG GAT **GAA** CTA ACA
Codon 581		
A*	Ala	A GGA TTT **GCG** AAG AAA CA
G	Gly	A GGA TTT **GGG** AAG AAA CA
Codon 613		
A*	Ala	GA TTT ATT **GCC** CAT TGC
T	Thr	GA TTT ATT **ACC** CAT TGC
S	Ser	GA TTT ATT **TCC** CAT TGC

[a] Entries in column 3 indicate the amino acid changes that occur at the relevant codons following the point mutation. The sequences in bold represent the codon within which the point mutation occurs.

[b] *, wild-type sensitive codon.

K540E mutations being the most frequently reported (12, 41, 42).

The role of the point mutations at each locus in conferring resistance to SP in vivo has been inferred from studies showing predictive association of particular mutations with treatment failure (21, 28) and from overrepresentation of mutations in recrudescent infections after treatment (2, 3, 8, 10, 11, 13, 16–18, 20, 26). Such studies are complicated by mixed infections. As the blood-stage parasites are haploid, the cooccurrence of two or more genotypes in an infection means that variation at multiple sites cannot be assigned to an individual parasite line within the infection and, accordingly, that the predictive association with treatment outcome is rendered less straightforward.

To study the frequency of alleles at a population level, we have designed a PCR high-throughput approach which uses sequence-specific oligonucleotide probes (SSOP) for detection of known single nucleotide polymorphisms (SNP) to identify and construct haplotypes. Haplotypes are combinations of SNP that are in the same gene in the same parasite, as distinct from associations of point mutations that cooccur because there is a mixture of parasites of different genotypes within a single infection. Haplotypes are biologically meaningful, since they determine the resistance properties of parasites that are exposed to drugs at the time of treatment. For example, a triple-mutant *dhfr* haplotype of N51I+C59R+S108N has 1.5- to 3-fold-higher pyrimethamine resistance in vitro than either the N51I+S108N or C59R+S108N double-mutant haplotype (35). A mixed infection containing these two double-mutant alleles is less resistant to pyrimethamine than an infection containing the triple-mutant allele, despite all three mutations being present in either case. When comparing populations, it is important to measure the frequency of haplotypes rather than the prevalence of each point mutation separately, because haplotypes are the determinants of resistance levels.

The method we have employed involves the PCR amplification of sequences from the coding regions of *dhfr* and *dhps* genes, which are fixed onto membranes and probed with SSOP (7) designed to detect each of the single base pair substitutions at all positions summarized in Table 1. The SSOP method has advantages for high throughput, while retaining sensitivity and specificity equivalent to those of other methods used for detection of *dhfr* and *dhps* SNP (1, 31). We used tetramethylammonium chloride (TMAC) to standardize the melting temperature of digoxigenin-labeled oligonucleotide probes, thus enabling duplicate membranes to be probed and washed at a standard temperature and sequence variants at all SNP sites to be detected simultaneously. Chemifluorescent signals from the detection of probes for differing SNP at the same site were compared quantitatively using a Storm phosphorimager. This high-throughput method allowed us to command data from large sample sizes.

MATERIALS AND METHODS

The study area and the samples. *P. falciparum*-positive samples were collected from two separate studies within the same area of northeast Tanzania; one was a study of infants and young children in Hai district, and the second was a study of people up to 45 years of age in the North and South Pare sites. Both studies were cross-sectional malariometric surveys across an altitude band of 550 to 1,600 m, and most of the study subjects were asymptomatic.

For the villages in Hai district, samples were collected in May 2001. All children less than 5 years old from 16 randomly selected villages were invited to attend the survey clinic at a central clinic. A finger-prick blood sample for blood slides and a filter paper blood sample were collected from each participating child. The filter paper blood samples were air dried and stored at 4°C with desiccant. Bloodspots from blood film-positive children were selected retrospectively for genotyping.

Samples from the North and South Pare Mountains were collected in November 2001 during malariometric cross-sectional surveys. A random sample of 1,250 individuals (250 per village) under 45 years of age was recruited, and a finger blood sample was taken into EDTA Microtainer tubes. Filter paper bloodspots were made with 10 μl of packed cells from samples of individuals found to be parasite positive.

Ethics. Scientific and ethical clearance for both studies was granted from the National Institute of Medical Research in Tanzania and the London School of Hygiene and Tropical Medicine. Consent was obtained from all individuals or their guardians prior to enrollment in the surveys.

TABLE 2. Table of PCR primer sequences and reaction conditions for the nested amplification of *dhfr* and *dhps*

Gene and primer	Primer sequence	PCR conditions
dhfr		
Outer, M1	5′ TTTATGATGGAACAAGTCTGC 3′	94°C × 3 min
650 bp, M7	5′ CTAGTATATACATCGCTAACA 3′	94°C × 1 min, 52°C × 2 min, 72°C × 1 min, 40×; 72°C × 10 min
Inner, M3b	5′ TGATGGAACAAGTCTGCGACGTT 3′	94°C × 3 min
594 bp, M9	5′ CTGGAAAAAATACATCACATTCATATG 3′	94°C × 1 min, 44°C × 2 min, 72°C × 1 min, 4×; 94°C × 1 min, 44°C × 1 min, 72°C × 1 min, 34×; 72°C × 10 min
dhps		
Outer, N1	5′ GATTCTTTTTCAGATGGAGG 3′	94°C × 3 min
770 bp, N2	5′ TTCCTCATGTAATTCATCTGA 3′	94°C × 1 min, 51°C × 2 min, 72°C × 1 min, 40×; 72°C × 10 min
Inner, R2	5′ AACCTAAACGTGCTGTTCAA 3′	As described above for *dhps* outer primer sequence
711 bp, R	5′ AATTGTGTGATTTGTCCACAA 3′	

DNA extraction. DNA extraction from bloodspots on filter paper was carried out in a 96-well plate format. A segment of the bloodspot was first soaked in 0.5% saponin–1× phosphate-buffered saline overnight and was then washed twice in 1 ml of 1× phosphate-buffered saline. The segment was then boiled for 8 min in 100 μl of PCR-quality water–50 μl of 20% Chelex suspension in distilled water (pH 9.5).

PCR amplification of *dhfr* and *dhps*. A 711-bp fragment of *dhps* and a 594-bp fragment of *dhfr* containing the polymorphic codons were independently amplified by nested PCR in a 96-well plate format. PCR primer sequences and reaction conditions are indicated in Table 2. The 25-μl PCR mix contained primers at 0.25 μM final concentration, 2 mM $MgCl_2$, 250 μM each deoxynucleoside triphosphate, and 1× Bioline *Taq* polymerase. Template DNA (1 μl) was introduced to outer reaction mixtures. The *dhps* outer PCR product (1 μl) was introduced into a 25-μl inner amplification mixture. Aliquots of 1 μl of threefold-diluted *dhfr* outer PCR product were introduced into a 25-μl inner amplification reaction mixture.

Use of SSOP for molecular genotyping of point mutations. Final-round PCR products were heat denatured (95°C for 2 min), cooled, and then spotted onto nylon membranes in 1-μl volumes in a 12 by 8 grid. A panel of four PCR samples of known sequences representing all common sequence variants was spotted on every blot to act as positive-negative controls for probe specificity. Replicate blots were made of each array so that simultaneous probing with the oligonucleotide probes of the full panel for that gene could be conducted. After drying, cross-linking was performed with 1,200-J UV light. Sequence-specific 18-bp oligonucleotide probes 3′ end labeled with digoxigenin (DIG) (Roche Boehringer Mannheim, Mannheim, Germany) were each designed to complement the known sequence polymorphisms in *dhfr* and *dhps* listed in Table 1. SNP-specific hybridization was followed by high-stringency TMAC washes, and alkaline phosphatase-conjugated anti-DIG Fab fragments (Roche Boehringer Mannheim) were used to detect DIG-labeled probes as described by Conway et al. (7). Visualization was performed through the alkaline phosphatase-catalyzed breakdown of the fluorogenic substrate ECF (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom) and scanned on a Molecular Dynamics Storm 840 PhosphorImager (Amersham Pharmacia Biotech).

Scoring. We scored the presence, absence, or relative abundance of the variant sequence polymorphism at each site separately. Images of blots probed with variant sequences for a single locus were transferred as TIF files to ImageMaster Total Lab software (Amersham Pharmacia Biotech). In the array analysis subsection of the software, a standard area of each spot was defined and the intensity of chemifluorescence in that area was measured. Background data were adjusted for by subtraction of the volume of the negative controls from the volume data. Thus, the volume of chemifluorescence for each spot was calculated as Volume = (maximum intensity × spot area) − background. To determine the threshold of detection per se, the presence-flagging option was employed. By this method, the faintest spot considered present and not background was selected to set the flagging threshold value. To compare SNP-specific probes at a single site, Microsoft Excel bar charts were drawn comparing the volume data for each probe on every sample to the presence-flagging result in each case. The following rules were used to determine whether a SNP was present or absent at each site.

(i) An SNP was considered present in a PCR product when the volume value with a particular probe was higher than that of the background. When volume values were low, presence flagging provided an internal control for avoiding possible biasing between probes or blots.

(ii) An SNP was considered absent when all volume values below the first gridline on the chart were rejected. No set value can be given for this criterion, as volume value comparison is relative and differs depending on the strength of the probe labeling and binding.

Samples were categorized into the single, majority, or mixed category at each site as follows. Samples were considered to be of mixed haplotypes when the volume value of the minority SNP was more than half the volume value of the majority SNP. Samples were considered to be mixed but containing a majority SNP when the minority SNP value was less than half of the majority value but higher than that represented by the first gridline on the chart. Samples were considered to be single when only one SNP was present at a site according to the rules given above.

To combine data from all sites in a gene and construct haplotypes, it was necessary to discard samples in which a mixture was found but which did not contain a majority SNP. Thus, for the purpose of generating frequency data, one haplotype was scored from each sample; this was either a single or majority type, because haplotypes from mixed infections cannot be constructed.

Following the same principle, for measuring the frequency of two-locus genotypes, a subset of samples in which a single or majority allelic haplotype was found at both *dhfr* and *dhps* was used.

Statistical analysis. Statistical analysis of population differences in haplotype frequencies (Wright's F_{st} [fixation index]) (6) and linkage disequilibrium (LD), given as D′ coefficient, was carried out using Arlequin software (33). Statistical analysis of contingency tables of the association of haplotypes within two-locus combinations was performed using a χ^2 test.

RESULTS

Of the 165 bloodspots that yielded PCR products, 10.3% were mixed at *dhps* and 1.2% were mixed at *dhfr*; no majority haplotypes were found. The low number of mixed infections was a reflection of the low level of transmission in the area. On stratification of the populations of the North Pare and South Pare Mountains into the age ranges of 0 to 4 and 5 to 45 years, no significant difference was found in the frequencies of *dhfr* and *dhps* allelic haplotypes, allowing comparisons to be made between the samples from those sites and those from Hai district, where samples were taken exclusively from subjects <5 years of age.

The *dhfr* and *dhps* allelic haplotypes present in each region are shown in Fig. 1. Three point mutations were found in *dhfr*, and of the eight possible haplotypic conformations, five were found; this matches well with findings of point mutations present in single-genotype infections elsewhere in East Africa that have been described previously (26, 41). Three point mutations were likewise found in *dhps*, and of the eight possible haplotypes, only three were found in *dhps*.

Regional haplotype frequencies are summarized in Fig. 2. Not all alleles at *dhfr* and *dhps* were present in each of the

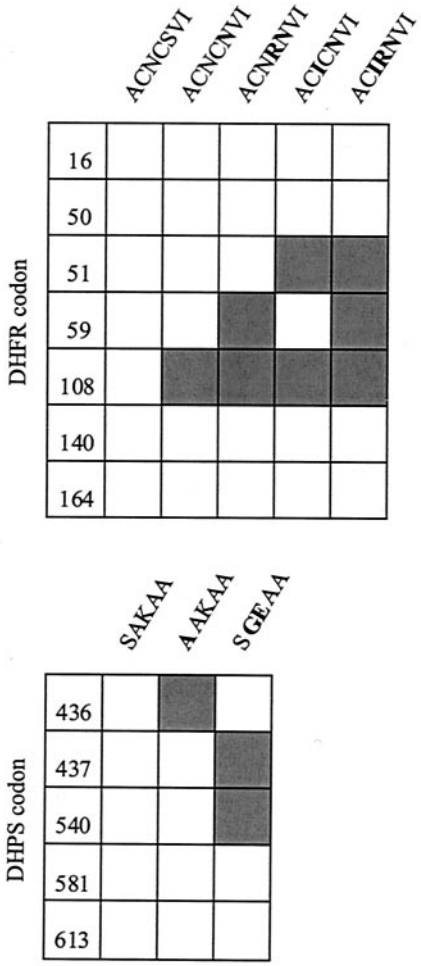


FIG. 1. Scheme of *dhfr* and *dhps* alleles found in this study. Shaded blocks indicate the sites at which the constituent SNP of each haplotype arise. Names of the alleles are composed of the amino acids present at each of the sites, in consecutive order by codon number, that are described as having a role in SP resistance. Substitutions are in boldface characters.

districts. The sensitive *dhfr* allele was not present in Hai district or the South Pare Mountains. The frequency of the *dhfr* triple-mutant allele was high in all districts and was highest in Hai (84.1%). The North Pare Mountains region had the lowest frequency of *dhfr* triple-mutant alleles; nevertheless, that frequency was five times greater than the frequency of sensitive *dhfr* alleles. We found no *dhfr* quadruple-mutant alleles (N51I+C59R+S108N+I164L).

The frequency of the *dhps* double-mutant allele was also greatest in Hai (64.2%) and lowest in the North Pare Mountains (43.3%); the frequency in the South Pare Mountains was intermediate (54.5%). Unlike that of *dhfr*, the sensitive allele of *dhps* was found in all districts. Furthermore, there was a low frequency (4 to 7.4%) of the single-mutant (S436A) allele in all districts.

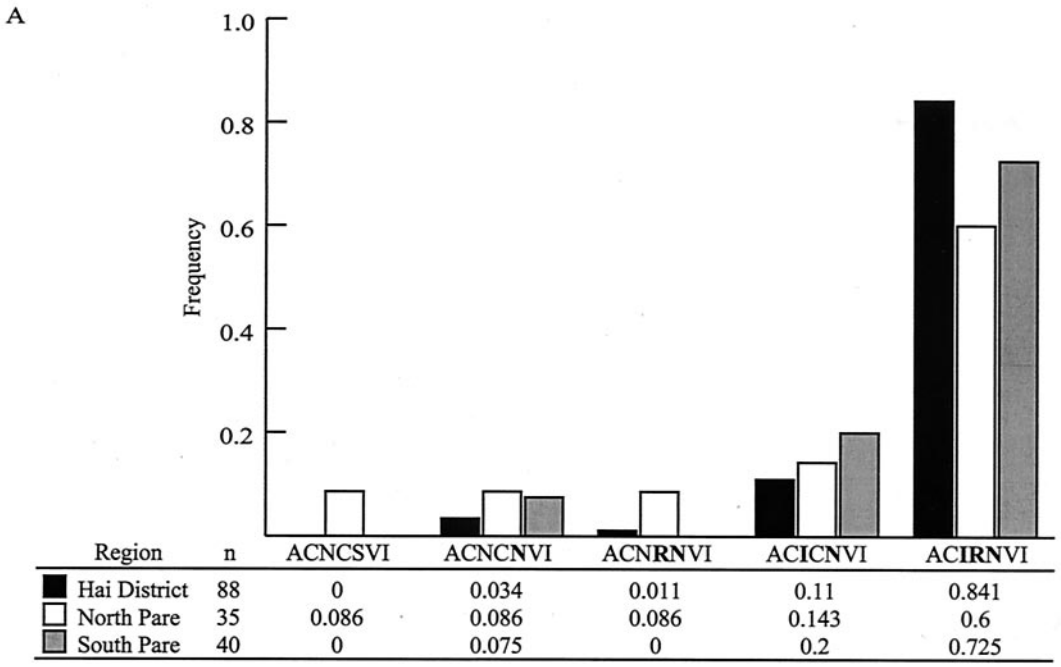
On calculation of the Wright's F_{st} (fixation index), the pairwise difference in haplotype frequencies at both loci between regions was shown to be statistically significant in comparisons of Hai district with the North Pare Mountains (*dhfr* F_{st} = 0.0733 [$P < 0.05$]; *dhps* F_{st} = 0.0748 [$P < 0.05$]). Interestingly, there was no significant difference in the remaining pairwise comparisons, despite the differences in haplotype frequencies between the North and South Pare Mountains.

Two-locus combinations were derived from single infections for which only one haplotype was recorded in both *dhfr* and *dhps*. The map in Fig. 3 shows the spatial distribution of frequencies of two-locus combinations. The reduction in sample size (n) reflects the loss due to mixed infections. The frequency of the most highly resistant genotype triple-mutant *dhfr*/double mutant *dhps* (ACIRNVI-SGEAA; substitutions are underlined) was found to be extremely high. The frequency was 63.2% in Hai district, 50% in the South Pare Mountains, and 22% in the North Pare Mountains. In the North Pare Mountains, there was more diversity at both loci and consequently a greater diversity of 2 locus genotypes. The Wright's F_{st} comparing the three populations at both loci further confirms the differences between the North and South Pare populations (F_{st} = 0.0583 [$P = 0.0054$]) and between the North Pare Mountains and Hai district (F_{st} = 0.149 [$P < 0.00001$]). Pairwise comparison of the South Pare and Hai district populations (F_{st} = 0.011) showed no significant difference and were merged for subsequent analysis. Statistical analysis of observed and expected two-locus combinations was performed on the population of the North Pare Mountains and the combined populations of Hai district and the South Pare Mountains. We found a significant departure from expected in the merged Hai-South Pare Mountains population (χ^2 test [$P = 0.0018$, 6 df]), whereas the distribution in North Pare was nonsignificant (χ^2 test [$P = 0.835$, 8 df]). LD analysis was performed on the combined Hai and South Pare Mountain data set, and we found three two-locus combinations to be in LD, namely, ACICNVI-SAKAA ($D' = 0.277$, $P = 0.01$), ACNRNVI-AAKAA ($D' = 1.0$, $P < 0.00001$), and ACIRNVI-SGEAA ($D' = 0.229$, $P = 0.031$) (substitutions are underlined). No other pair of alleles was found to be in LD.

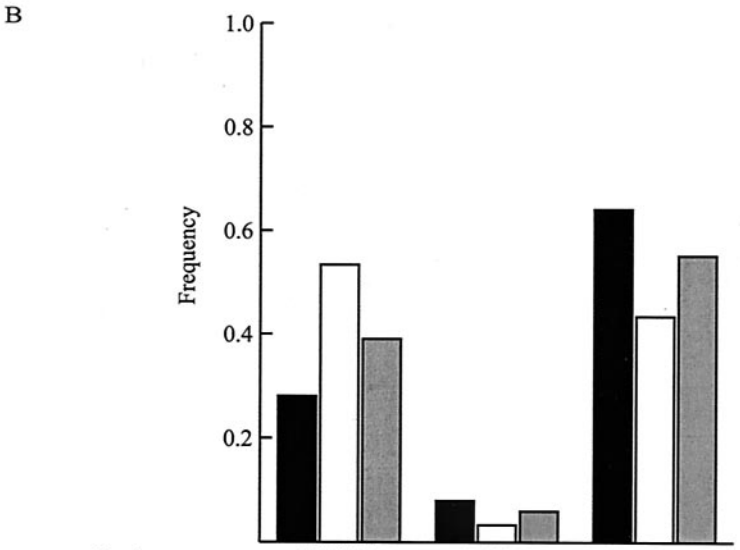
DISCUSSION

We have described a new approach by which blood survey material was used to determine the frequency of point mutation haplotypes in *P. falciparum* populations. This approach allows quantitation of resistance at the population level and enables direct comparison of population resistance levels even when they differ widely in the proportions of multiply infected individuals. The issue of multiple infections can be problematic when genotyping blood-stage parasites, because it causes haplotypic conformations of point mutations to be obscured and rare mutations to be oversampled. By recording one genotype per infection and discounting minority genotypes, we were able to avoid the oversampling of rare genotypes and to estimate the frequency of mutation haplotypes in the population in a manner which was standardized over all populations of different transmission intensities. With this consideration in mind, the SSOP method employed is designed for high-throughput screening of blood-stage infections to derive haplotype frequencies from survey material.

We have shown that only a subset of point mutations reported globally were present in North Tanzania in these populations, confirming previous reports of analyses of natural *P. falciparum* populations of East Africa (26, 41). Using single-



Region	n	ACNCSVI	ACNCNVI	ACNRNVI	ACICNVI	ACIRNVI
Hai District	88	0	0.034	0.011	0.11	0.841
North Pare	35	0.086	0.086	0.086	0.143	0.6
South Pare	40	0	0.075	0	0.2	0.725



Region	n	SAKAA	AAKAA	SGEAA
Hai District	81	0.284	0.074	0.642
North Pare	30	0.533	0.033	0.433
South Pare	33	0.394	0.061	0.545

FIG. 2. (A) Frequencies of *dhfr* alleles found in the district of Hai and the regions of the North and South Pare Mountains. (B) Frequencies of *dhps* alleles found in the district of Hai and the regions of the North and South Pare Mountains.

and majority-genotype infections, we were able to determine the point mutation haplotypes that were present in the three districts surveyed. We found five alleles at *dhfr*, and in reviewing the single-infection data from other studies in Africa, it is possible to see these same haplotypes in Kenya, Malawi, Tanzania, and Mauritania. We found three haplotypes in *dhps*, the sensitive-allele haplotype, the single-mutant allele S436A haplotype, and the double-mutant allele A437G K540E haplotype which has been widely recorded in East Africa (18, 19, 21, 25–27, 41) and not in West Africa (12, 30, 41).

Regional variation in population resistance. Allelic haplotype frequencies in the three regions differed significantly. Those of the Hai district and South Pare populations were highly resistant at *dhfr*, with triple-mutant allele (N51I+C59R+S108N) frequencies of >70% and *dhps* double-mutant allele (A437G+K540E) frequencies of >50%.

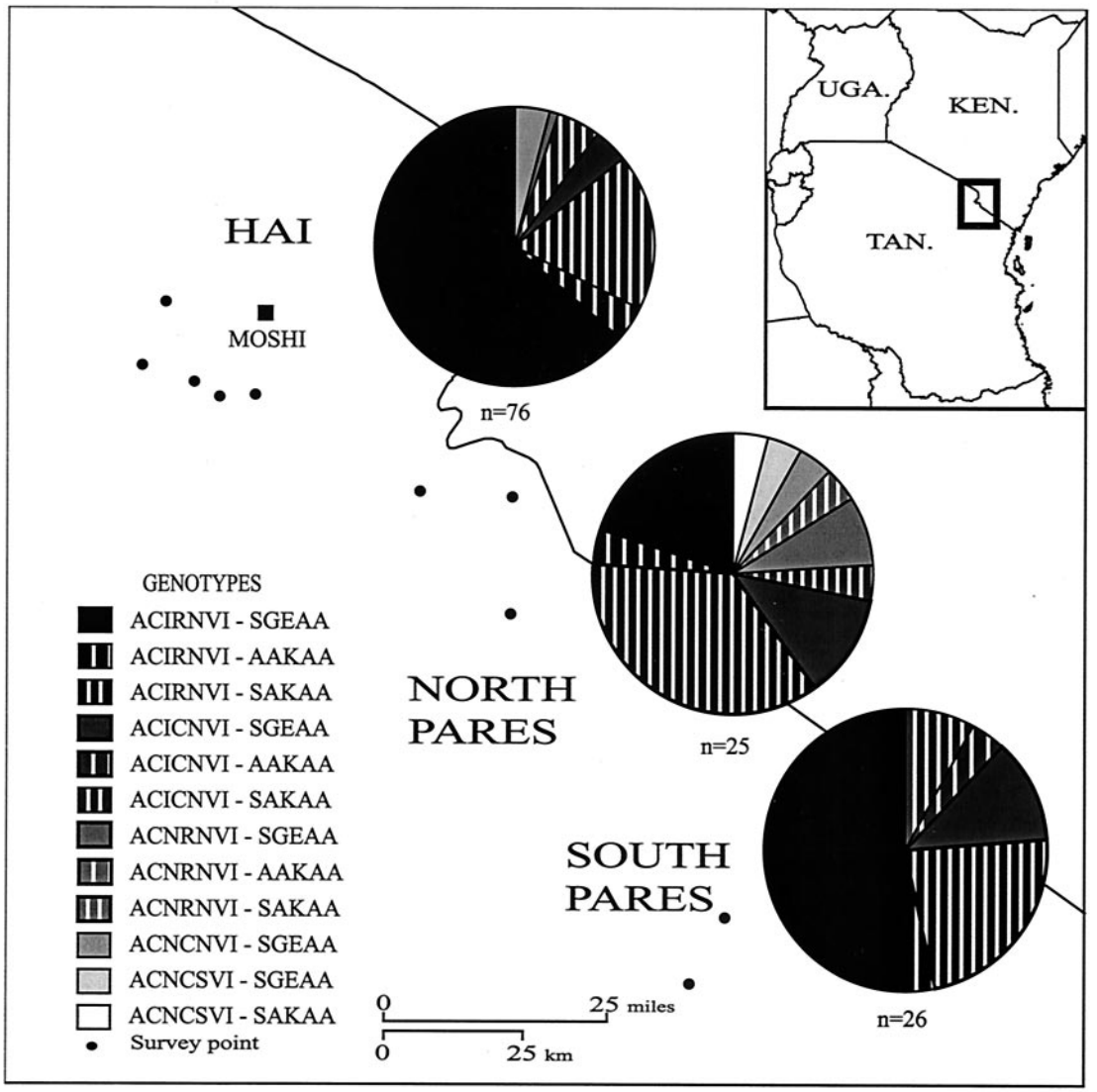


FIG. 3. Map of the three districts of northern Tanzania showing the frequencies and distribution of *dhfr*/*dhps* two-locus combinations. The genotype represented by the open symbol contains both sensitive alleles, and that represented by the black symbol contains the most resistant alleles.

The same alleles were present in the North Pare population but at lower frequencies. Sensitive alleles at either locus were absent in the Hai district and South Pare populations but were present at low frequency in the North Pare population. The North Pare population was significantly distinct from the other two populations, with the most significant difference found between those of the North Pare and Hai district populations. These findings point to a slower development of SP resistance in the North Pare Mountains. Possible causes of weaker selective pressure are greater use of alternative antimalarials such as amodiaquine and reduced use of antimalarials per se due to poor access to healthcare facilities or raised levels of acquired immunity. Such differences are maintained in the face of gene flow, which operates to make the parasite populations more homogeneous. Ongoing investigations into treatment-seeking behavior and overprescription by providers aim to further illuminate differences in drug use among the three study populations (T. Swarthout, D. Chandramohan, F. Mosha, A. Bell, G. Masuki, C. Drakeley, and H. Reyburn, Abstr. 3rd MIM Pan-African Malaria Conf., 2002).

Recent work in real populations in Malawi has found statistical association of the presence of all three *dhfr* mutations and both *dhps* mutations with failure to clear parasitemia after SP treatment (21). It is probable that in many cases this is due to the presence of the two most highly resistant alleles, triple *dhfr* and the double *dhps*—and this idea is supported by analysis of recrudescent infections following SP treatment (22). Here we directly measured the frequency of the highly resistant two-locus genotype consisting of the ACIRNVI-SGEAA (substitutions are underlined). We found that frequencies were twice as high in Hai district as in the South Pare Mountains and three times as high as in North Pare Mountains; hence, there is a clear and testable indication that SP treatment failure rates in these three regions are likely to differ.

Selection on *dhfr* and *dhps* by SP use. It has been observed that *dhfr* mutations appear earlier than *dhps* mutations in the development of SP resistance within populations in Africa (23, 26, 34). Our own data suggest the same sequence of events, with resistant *dhfr* being fixed in Hai and the South Pare Mountains yet with sensitive *dhps* being relatively common. The observed LD between the ACICNVI-SAKAA and the absence of significant LD between the *dhfr* double mutants and the *dhps* double-mutant alleles supports the idea of an interaction between *dhfr* and *dhps* in the development of resistance.

Furthermore, there was a statistically significant association between the triple *dhfr* mutant and the double *dhps* mutant in the combined Hai district and the South Pare populations. *dhfr* is on chromosome 4 and *dhps* is on chromosome 8, so the fact that LD was found between two highly resistant haplotypes at these unlinked loci is indicative of the nonindependence of SP selection. This relationship has been reported previously in Kenya (28) and Malawi (21). The same relationship was not apparent in North Pare, where the frequency of resistance alleles at both loci was also significantly lower; both this and the absence of statistically significant LD imply that drug selection is weaker. This finding emphasizes the transient nature of linkage, particularly in areas of high transmission intensity, where recombination rapidly breaks down the linkage between *dhfr* and *dhps*, and argues for caution in the use of indicator mutations as a proxy for resistance genotyping. While the cooccurrence of all five mutations in an infection in the South Pare Mountains and Hai district was in fact a reliable indicator of the presence of a quintuple genotype, this was not the case in the North Pare Mountains. The widespread use of the antibiotic Septrin, which contains trimethoprim and sulfamethoxazole, to treat other infections may indirectly select for *dhfr* and *dhps* resistance mutations and further complicate the relationship.

Past, present and future of pyrimethamine and sulfadoxine use in northern Tanzania. The three districts described here are found in an area between 200 km and 400 km northwest of Muheza district, a region historically associated with exceptionally high levels of antimalarial drug resistance. Resistance to pyrimethamine alone was reported in 1954 in Mngeza in Muheza district, following mass administration of prophylactic doses of pyrimethamine monotherapy over a five-month period during 1953 (5). Use of the SP combination began in Tanzania in 1982, when it was introduced as a second-line treatment for use in cases of chloroquine failure, and as early as 1984 it was the policy of the Muheza district hospital to use SP as a first-line antimalarial (25). Studies from the region report that SP was highly effective during the eighties, but resistance was recorded in Magoda village near Muheza in 1994 (32) and was subsequently reported in villages in the surrounding area (18, 19, 37). The emergence of resistance to SP in 1994 was attributed by the authors to be in part a result of the prophylactic intervention with weekly dapsone pyrimethamine treatment to all children less than 10 years old. Resistance to SP in Muheza district hospital is now reported to be as high as 45% (25).

Two explanations for the high frequency of resistance alleles in the three districts described here are the widespread use of SP or related drugs and the movement of resistance from Muheza. However, levels of resistance do not show a simple decline with distance from Muheza to Hai (Fig. 3), and drug use is clearly a very important factor. What is striking from the data presented here are the significant interpopulation differences, and it is probable that these differences have arisen from differing patterns of drug use in these communities.

In this paper we have described a new technique for detection of resistance point mutations in *dhfr* and *dhps*. We have argued for the use of haplotype frequencies as a measure of resistance at the population level and as an appropriate tool for describing the spread of resistance. In displaying the power of this technique, we have shown significant regional heterogeneity and identified a region of northern Tanzania where resistance to SP is already unprecedentedly high. Since policy recently mandated a switch to use of this drug as the first-line treatment, we recommend that in vivo studies be performed to confirm that the high frequency of resistance alleles is indicative of treatment failure, as is predicted on the basis of studies conducted elsewhere in Africa.

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